Synergistic interactions between volicitin, jasmonic acid and ethylene mediate insect-induced volatile emission in *Zea mays*

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Plants display differential responses following mechanical damage and insect herbivory. Both caterpillar attack and the application of caterpillar oral secretions (OS) to wounded leaves stimulates volatile emission above mechanical damage alone. Volicitin (*N*-17-hydroxylinolenoyl-L-glutamine), present in beet armyworm (BAW, *Spodoptera exigua*) OS, is a powerful elicitor of volatiles in excised maize seedlings (*Zea mays* cv. Delprim). We consider some of the mechanistic differences between wounding and insect herbivory in maize by examining the activity of volicitin, changes in jasmonic acid (JA) levels, and volatile emission from both intact plant and excised leaf bioassays. Compared to mechanical damage alone, volicitin stimulated increases in both JA levels and sesquiterpene volatiles when applied to intact plants. In a bioassay comparison, excised leaves were more sensitive and produced far greater volatile responses than intact plants following applications of both volicitin and JA. In the excised leaf bioassay, volicitin applications (10–500 pmol) to wounded leaves resulted in dose dependent JA increases and a direct positive relationship between JA and sesquiterpene volatile emission. Interestingly, volicitin-induced JA levels did not differ between intact and excised bioassays, suggesting a possible interaction of JA with other regulatory signals in excised plants. In addition to JA, insect herbivory is known to stimulate the production of ethylene. Significant increases in ethylene were induced only by BAW herbivory and not by either wounding or volicitin treatments. Using intact plant bioassays, ethylene (at $1 \, \mu l \, l^{-1}$ or less) greatly promoted volatile emission induced by volicitin and JA but not mechanical damage alone. For intact plants, wounding, elicitor-induced JA and insect-induced ethylene appear to be important interacting components in the stimulation of insect-induced volatile emission.

Introduction

Plant physiological responses following insect herbivory and simple mechanical damage are not the same (Baldwin 1988, Turlings et al. 1990, Korth and Dixon 1997, Reymond et al. 2000). The demonstration that plants can perceive insect attack as being different from wounding has generated considerable interest in the elucidation of underlying mechanisms that regulate insect-induced plant defenses (Karban and Baldwin 1997). Differences in the spatial and temporal nature of the mechanical damage may be important. However, few attempts have been made to accurately compare insect herbivory and similarly mimicked mechanical damage. In cases where careful controls have been performed, insect damage still resulted in significantly different plant responses (Baldwin 1988, McCloud and Baldwin 1997). Insect-derived elicitors and enzymes that contact the plant leaf surface during feeding are now considered probable candidates to explain the specificity of plant responses to insects (Mattiacci et al. 1995, Alborn et al. 1997, Felton and Eichenseer 1999).

One rapid and dramatic example of an insect-specific plant response is induced-volatile emission in maize

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Abbreviations – JA, jasmonic acid; BAW, beet armyworm (*Spodoptera exigua*); OS, oral secretions.
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(*Zea mays*) seedlings triggered by Noctuid caterpillar herbivory. In this system, indole and a range of sesqui-terpene volatiles become major headspace components only a few hours after feeding by the beet armyworm (BAW; *Spodoptera exigua*), whereas only trace levels of volatiles are released following mechanical damage alone (Turlings et al. 1990). Insect-induced plant volatiles function as reliable and specific chemical signals for the attraction of parasitoids and predators, the natural enemies of attacking insect herbivores (Turlings et al. 1990, Vet and Dicke 1992, DeMoraes et al. 1998, Kessler and Baldwin 2001). Induced volatile emission can be stimulated by applying caterpillar oral secretions (OS) to wound sites on the plant leaf surface (Turlings et al. 1990, 1993). Volicitin (*N*-17-hydroxylinolenoyl-L-glutamine) has been isolated and identified from BAW OS as a highly active elicitor of volatile emission in excised maize seedling bioassays (Alborn et al. 1997, Schmelz et al. 2001). Additional fatty acid-amino acid conjugates with volatile inducing activity are now known to occur in the OS of other caterpillar species (Paré et al. 1998, Pohnert et al. 1999, Alborn et al. 2000, Halitschke et al. 2001).

The mechanism by which insect herbivory and insect-derived elicitors promote induced volatile production is an active area of research. Early work with exogenous additions of jasmonic acid (JA) to excised leaf bioassays suggested that jasmonates might be important signals in regulating insect-induced volatile emission (Hopke et al. 1994, Boland et al. 1995). Likewise, the linolenic acid moiety of volicitin gave early clues of its probable involvement with the plants octadecanoid-signalling pathway (Alborn et al. 1997, Paré and Tumlinson 1999). The first evidence that factors present in caterpillar OS influence wound hormone levels, such as JA, came from research by McCloud and Baldwin (1997) while examining inter-actions of *Manduca sexta* with *Nicotiana sylvestris*. A rapid and transient increase in the induced levels of foliar JA was detected when *M. sexta* OS was applied to mechanical damage sites. More recently, Halitschke et al. (2001) demonstrated that a combination of fatty acid-amino acid conjugates, present in *M. sexta* OS, were sufficient to induce both JA and volatiles when applied to wounded tobacco (*Nicotiana attenuata*) leaves.

It is well established that JA pools increase following mechanical damage in plant tissues (Creelman and Mullet 1997); however, mechanical damage alone usually results in little or no induction of volatiles (Turlings et al. 1990, 1993). This raises the question 'Why doesn't mechanical damage alone induce significant volatile emission?'. Subtle yet significant differences in the magnitude, duration, or subcellular localization of the induced JA levels may exist following insect herbivory and mechanical damage. Also, significant interactions may exist between additional plant hormones that are also influenced by either mechanical damage or insect feeding. In solanaceous plants, wounding alone alters both abscisic acid (Peña-Cortés et al. 1989) and indole acetic acid levels (Thornburg and Li 1991), while *M. sexta* herbivory triggers a burst in ethylene emission (Kahl et al. 2000). In tobacco, additions of either indole acetic acid or ethylene

are known to greatly influence wound and insect-induced nicotine accumulation that is regulated by jasmonates (Baldwin et al. 1997, Kahl et al. 2000). These findings make it likely that the levels of various endogenous hormones will influence insect-induced volatile emission.

Maize seedlings were one of the first bioassay systems used to demonstrate rapid changes in plant chemistry where caterpillar feeding and treatments with insect OS resulted in dramatically different induced responses compared to wounding alone (Turlings et al. 1990). This system has since been widely used to probe the activation of secondary pathways (Degenhardt and Gershenzon 2000, Frey et al. 2000, Shen et al. 2000), and nature of plant–insect interactions mediated by volatiles (Hoballah and Turlings 2001). We now examine the role of the insect-derived elicitor, volicitin, and JA as potential mediators of the differential plant responses generated following mechanical damage and insect herbivory. Using a single dose, we previously demonstrated that intact plants display a dramatically lower induction of volatiles following the application of volicitin when compared to excised leaves (Schmelz et al. 2001). This point is not trivial as it emphasizes the requirement of carefully designed bioassays to realistically simulate herbivory in field studies that attempt to understand the ecological significance of volatiles as induced-plant defense responses. In an effort to account for these differences in elicitor activity we use both intact plant and excised leaf bioassays to quantify the role of wounding and volicitin in modulating both JA levels and plant volatile emission. In addition to JA, insect herbivory is also known to increase ethylene emission (Kahl et al. 2000, Schmelz et al. 2003) thus we examine effect of the wounding, volicitin and BAW herbivory on ethylene production. Finally, we consider the interactions of ethylene with both volicitin and JA treatments to examine how these hormones together may influence volatile emission during actual herbivory.

Materials and methods

Plant growth and insect rearing

Seeds of *Z. mays* L. cv. Delprim were acquired from Delley Seeds and Plants Ltd. (Delley, Switzerland), germinated in potting soil and transferred to hydroponic containers after 6 days (see Schmelz et al. 2001). All plants were maintained in a 12-h photoperiod (0600–1800 h) with 350 μmol m^{-2} s^{-1} of photosynthetically active radiation supplied by a mixture of both high pressure sodium and metal halide lamps (400 W, GE Lucalox®) with 70% relative humidity and a temperature cycle of 22°C/26°C (night/day). Treatments and volatile analysis were performed on 10- to 12-day-old plants that contained 3–4 leaves.

Experimental designs and treatments

For mechanical damage treatments, each of the oldest three leaves of individual plants received two superficial

damage sites using a razor to scratch the abaxial surface of the leaves perpendicular to, but not including, the midrib vasculature. The mechanical damage sites (normally 2 mm × 10 mm) were approximately equidistant between the base and tip of the leaf but laterally staggered by 2 cm with one on each side of the midrib. This treatment disrupted the waxy cuticle and epidermal cells and allowed applied buffer solutions to cling to the leaf surface. A total of either 10 or 18 µl of 50 mM $NaPO_4$ (pH = 8.0) buffer was distributed evenly between all mechanical damage sites on each plant immediately after wounding. The quantity of either volicitin or JA applied to each wounded plant is specified in each experiment. Twenty minutes after the mechanical damage treatments, leaves from groups designated as 'Excised' were cut from intact plants at the base of the petiole and placed into 4-ml vials containing purified H_2O. Intact plants were not manipulated further. In the maize seedling system, the quantities of induced volatiles emitted change dramatically throughout the light cycle (Schmelz et al. 2001); thus, both the time of elicitation and volatile collection are specified for each experiment. Differences in timing resulted in quantitative differences in reported volatile emission between experiments.

Time course of volicitin induced JA and volatiles. For the intact plant time course, plants were either left untreated or mechanically damaged and treated with either 18 µl of buffer or buffer containing volicitin (5 nmol). Plants from these 3 groups were treated between 0930–1030 h and harvested for JA analysis by snap freezing in liquid N_2 at 5, 15, 30, 45, 60 and 120-min intervals. On an additional set of plants (n = 3), volatiles were collected for 4 h in the photoperiod between 3 and 7 h after treatment. Due to the timing of events only one plant (n = 1) per group could be managed at each time interval for JA analysis. Thus the entire experiment was repeated 4 times and the data from 108 plants was combined.

Effect of bioassay design on tissue sensitivity and JA induction. The sensitivity of intact plant and excised leaf bioassays to volicitin and JA were compared using the mechanical damage protocol. Immediately after wounding, volicitin and JA were applied to intact plants (n = 6) in 10 µl buffer at the levels of 0.0, 0.05, 0.5, 5.0 and 0.0, 0.5, 5, 50 nmol plant^{-1}, respectively. Within 20 min, treatments were split and leaves were either excised (n = 3) or plants left intact (n = 3). These 16 groups were designated as Excised and Intact, respectively. Leaf treatments occurred at 20:00 h and volatiles were collected in the following photoperiod for 4 h between 0900–1300 h. Possible interactions of leaf excision on volicitin-induced JA levels were then examined. Three treatments, performed at 20:00 h (n = 16) consisted of untreated controls, and mechanically damaged plants treated with either buffer alone or buffer plus volicitin (1 nmol plant^{-1}). Within 20 min, the 3 treatment groups were split and leaves were either excised (n = 8) or plants left intact (n = 8). After 2 h a subset of plants (n = 4) was harvested for JA analysis while volatiles (n = 4) were collected in the following photoperiod for 4 h between 0900–1300 h.

Relationship between volicitin, JA and volatiles. Using a sensitive excised leaf bioassay and a constant area of leaf mechanical damage, relationships between volicitin, induced JA and volatiles were examined. In this experiment the surface area of each wound site was reduced by half (i.e. 1 mm × 10 mm) to minimize the levels of wound-induced JA. Six treatment groups (n = 8) consisted of either undamaged controls or mechanically wounded leaves with 10 µl of buffer applied containing 0, 10, 30, 100 or 500 pmol volicitin. Plants were initially treated at 20:00 h. Two hours later, at 22:00 h, the 6 groups were split and half of the samples (n = 4) were immediately harvested for JA analysis. Plant volatiles were collected on the remaining leaves (n = 4) for 4 h, between 0900–1300 h, in the following photoperiod.

Interaction of volatile elicitors with ethylene. We examined the ability of volicitin to induce ethylene, as insect herbivory has been shown to increase ethylene production in multiple systems (Kendall and Bjostad 1990, Kahl et al. 2000). Using intact plants, four treatment groups (n = 4) were created, consisting of untreated controls, mechanical damage with buffer alone or buffer plus volicitin (1 nmol plant^{-1}), and BAW herbivory caused by placing 6 early 3rd instar BAW larvae per plant. In both intact plant and excised leaf assays, numerous preliminary trials looking for volicitin-induced ethylene production failed to demonstrate differences. To increase the chance of detecting a change, starting at 17:00 h intact plants were treated 4 times, every 5 h for 20 h. The repeated treatments were an effort to provide continued stimulus, as the BAW larvae were continually present on plants as a positive control for induction. Immediately after the final leaf treatments all plants were placed into sealed glass cylinders (127 ml) for the 1 h collection of headspace ethylene for analysis. We then examined the potential interaction of ethylene with volicitin and JA on the induced volatile emission of intact plants. For the volicitin trial, maize seedlings were wounded and treated with either buffer (n = 24) or buffer containing 1 nmol of volicitin (n = 24) at 17:00 h. Plants were then placed in individual glass jars (3.8 l) with 300 ml of hydroponic solution and sealed in with metal lids containing rubber septa ports. Each of these two treatment groups were subdivided into 4 additional groups (n = 6) by adding ethylene gas via a syringe to generate atmospheric concentrations of 0, 0.25, 0.5, 1 µl l^{-1}. The ethylene levels selected match those used to induce the well-characterized formation of aerenchyma in maize seedling roots (He et al. 1992, Drew et al. 2000). Plant volatiles were collected for 2 h at the beginning of the following photoperiod (06:00 h). In the JA trial, intact plants were wounded and treated with 10 µl of buffer containing either 0, 0.5, 5, or 50 nmol of JA at 17:30 h. Individual plants were then sealed in 3.8-l jars and treated with either no ethylene or 1 µl l^{-1} of atmospheric ethylene. Volatiles were collected from these eight treatment groups (n = 6) for 2 h at the beginning of the following photoperiod (06:00 h).

Analysis of plant volatiles and jasmonic acid

Collection and GC analysis of volatiles was performed as described in Schmelz et al. (2001). The term 'combined sesquiterpenes' is used to describe the summation of the three major insect-induced sesquiterpenes in maize (var. Delprim) namely β-caryophyllene (*E*)-α-bergamotene, and (*E*)-β-farnesene levels. GC/MS quantification of JA from plant tissues was modified from Weber et al. (1997) and follows directly from Schmelz et al. (2003). For pharmacological experiments, free JA was obtained from base hydrolysed methyl jasmonate (Sigma, St. Louis, MO, USA). The epimeric composition of this JA was determined to be 5.6% *cis* and 94.4% *trans* after diazomethane methylation and GC/MS analysis. Both *cis* and *trans* JA are known to possess biological activity; however, several studies indicate that *cis*-JA is more active than *trans*-JA (Beale and Ward 1998). Ethylene production from intact plants was determined by removing 1 ml of headspace from plant chambers (127 ml) temporarily sealed for 1 h. The headspace sample was analysed on an HP-5890 GC with injector, oven and flame ionization detector temperatures of 150, 80, and 250°C, respectively, and a HayeSep Q column (80/100 mesh, $6' \times 0.125' \times 0.085'$ I.D. Alltech, Deerfield, IL, USA) using a nitrogen carrier gas flow rate of $100 \, \text{ml} \, \text{min}^{-1}$. Quantification was based on an external standard curve constructed from 1-ml injections of known ethylene standards (Kao and Yang 1983). Calculations of ethylene production $(\text{nl} \, \text{g}^{-1} \, \text{h}^{-1})$ were based on the wet mass of the whole plant as headspace samples were removed from intact plants consisting of both roots and shoots.

Statistics

Analyses of variance (ANOVAS) were performed on the JA levels, combined sesquiterpenes, indole and ethylene. Significant treatment effects were investigated when the main effects of the ANOVA were significant ($P < 0.05$). Where appropriate, Tukey tests were used to correct for multiple comparisons between control and treatment groups. Dunnett's tests used to examine significant increases in treatment groups were compared to selected controls. Prior to statistical analysis all data was subjected to square root transformation to compensate for elevated variation associated with larger mean values (Zar 1996). The analysis was accomplished with JMP 3.0 statistical discovery software (SAS Institute Inc., Cary, NC, USA).

Results

Timing of wound and volicitin-induced JA

Volicitin promotes JA accumulation above mechanical damage alone. Within 5 min of mechanical wounding, foliar JA levels of intact plants increased from 2 to $23 \, \text{ng} \, \text{g}^{-1}$ FW (Fig. 1A). Between 15 and 60 min, wound-induced JA levels remained elevated at approximately $35 \, \text{ng} \, \text{g}^{-1}$ FW and then decreased to $19 \, \text{ng} \, \text{g}^{-1}$ FW by 120 min. The combination of leaf wounding and volicitin resulted in a sustained increase in JA with significantly greater levels than mechanical damage alone detected at both 45 min and 120 min (Fig. 1A). JA levels in undamaged control plants remained low and essentially unchanged during this experiment. Within the first 7 h of treatment, volicitin promoted a small yet significant 3.5-fold increase in sesquiterpene volatile emission above that of mechanical damage (Fig. 1B). Mechanically damaged plants treated with buffer displayed intermediate sesquiterpene volatile emission that was significantly higher than undamaged controls, yet lower than volicitin treated plants.

Comparison of intact plant and excised leaf bioassays

Following the treatment of leaves with both JA and volicitin, excision promotes both an increase in sensitivity to elicitation and quantitatively greater increases in volatile emission compared to the volatile responses of intact plants. On intact plants, 5 nmol of JA applied to
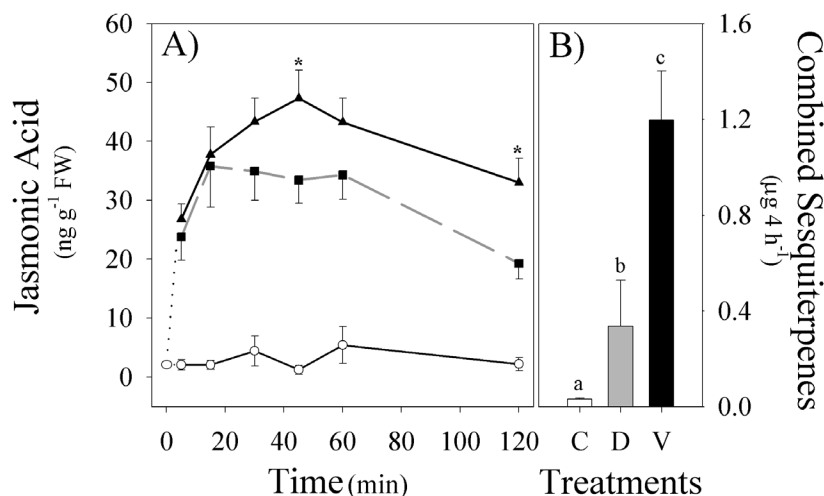

Fig. 1. Mean (± SEM) JA concentrations (A, n = 4) and combined sesquiterpene emission (B, n = 12) from intact maize seedlings treated in the morning. Treatments consisted of undamaged controls (C, ○) and damaged plants treated with either buffer (D, ■) or buffer + volicitin (V, ▲) at $5 \, \text{nmol} \, \text{plant}^{-1}$. Asterisks denote significant increases in JA above plants damaged and treated with buffer alone ($P < 0.05$, using Dunnett's test for defined multiple comparisons). Different letters (a, b, c) represent significant differences in volatile emission between treatments ($P < 0.05$, Tukey correction for multiple comparisons).

wounded leaves fails to induce sesquiterpenes, yet this level is sufficient to elicit volatile emission when a leaf is excised (Fig. 2A,B). Likewise, 0.05 nmol of volicitin has little activity on intact plants, but produces a significant increase in sesquiterpene volatiles when leaves are excised (Fig. 2C,D). Additionally, excised-leaves release quantitatively more volatiles and display greater fold differences between treatments than intact plants. Compared to appropriate wound plus buffer treatments, 50 nmol of JA stimulates a 4.3-fold increase in intact plants and a 8.3-fold increase in excised leaves (Fig. 2A,B). Likewise, 5 nmol of volicitin stimulates a 8.2-fold increase in intact plants and a 33.1-fold increase in excised-leaves (Fig. 2C,D). Thus excised-leaves respond to lower doses of JA and volicitin, produce quantitatively more volatiles, and exhibit greater magnitude differences between wounding and elicitation treatments.

Volicitin induces comparable levels of JA in both intact and excised leaf bioassays. Despite these similar changes in wound and volicitin induced JA levels, excision results in far greater sesquiterpene volatile emission. For intact plants, JA levels of untreated control, mechanical damage + buffer, and mechanical damage + volicitin treatments averaged 0.9, 11.0, and 25.3 ng g^{-1} FW, respectively (Fig. 3A). These JA levels were not significantly different from the corresponding values found in the excised leaf bioassay (Fig. 3A). Despite the

lack of differences in JA, the total amount of volicitin induced volatiles emission from the excised leaves was 4.3-fold greater than from intact plants (Fig. 3B). Thus within bioassays, induced levels of JA precede induced volatile emissions. However, JA levels alone do not explain the differences in volatile emission between intact plant and excised leaf bioassays. Also the low level of volatile emission from intact plants treated with volicitin suggests that the excised leaf bioassay is better suited for examining relationships between the stimulus, JA as a putative intermediary, and volatile emission response.

Volicitin, endogenous JA and volatile emission

In the excised leaf assay, increasing amounts of volicitin resulted in stepwise increases in JA accumulation. On a mean basis, wounding increased tissue JA levels from 0.5 to 5.2 ng g^{-1} FW. However, the first significant increase in JA occurred following the treatment of wounded leaves with 10 pmol volicitin and resulted in 10.5 ng g^{-1} FW of JA (Fig. 4A). Treatment of excised leaves with 500 pmol of volicitin resulted in 22.0 ng g^{-1} FW JA, significantly more than detected in leaves treated with 10 pmol. Sesquiterpene volatile emission the following photoperiod, largely mirrored the dose dependent increases in JA levels measured 2 h after treatments. Mechanical damage alone did not significantly induce
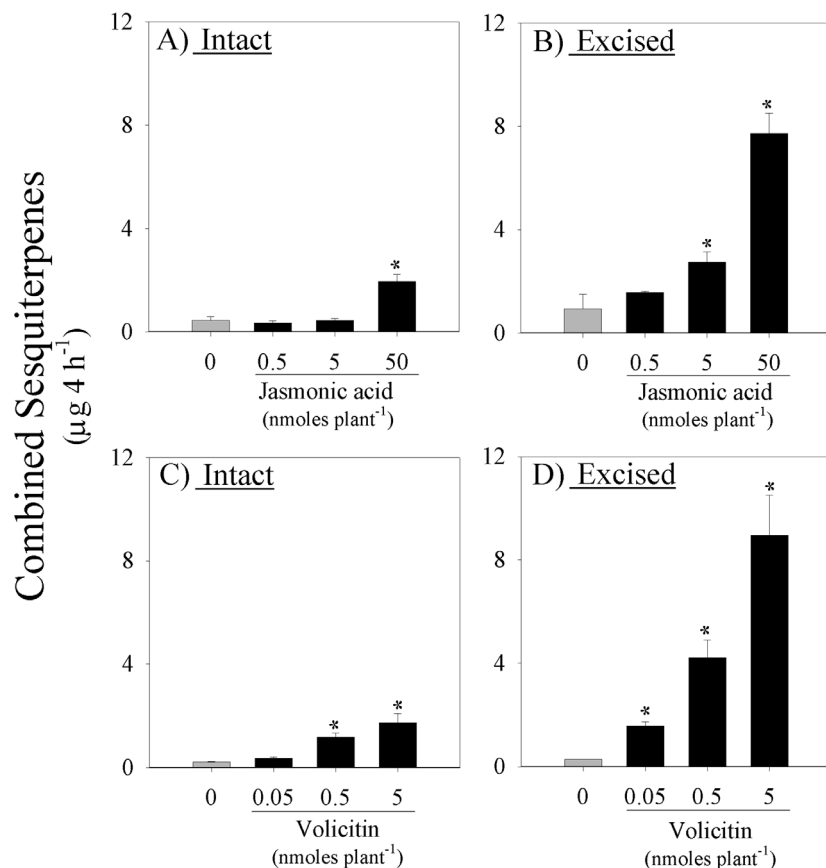

Fig. 2. Mean (+ SEM) combined sesquiterpene volatile emission of intact plant (A, C) and excised leaf (B, D) assays following leaf damage and treatment with either buffer only (0), volicitin (0.05, 0.5, 5 nmol plant^{-1}), or JA (0.5, 5, 50 nmol plant^{-1}). Asterisks denote significant increases in volatiles above the (0) damage + buffer treatment ($P < 0.05$, using Dunnett's test for defined multiple comparisons).
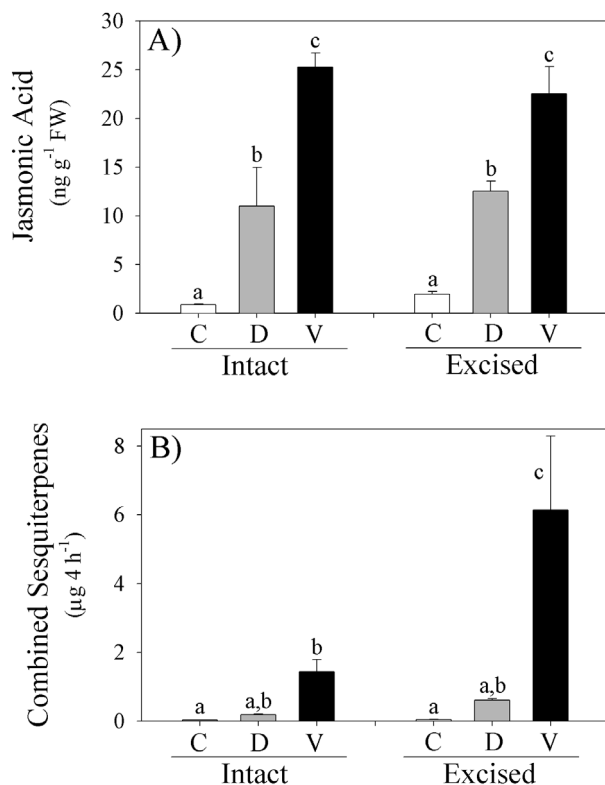
Fig. 3. Mean (+ SEM, n = 4) JA levels (ng g^{-1} FW) (A) and combined sesquiterpene emission (μg 4 h^{-1}) (B) of intact plants and excised-leaves that were left as untreated controls (C) or damaged and treated with either buffer only (D), or buffer plus volicitin (V) at 1 nmol plant^{-1} (A). Within figures, bars not sharing the same letters (a-c) represent significant differences (P < 0.05, Tukey correction for multiple comparisons).

sesquiterpene volatiles above undamaged controls. However, all concentrations of volicitin used stimulated volatile emission above undamaged controls (Fig. 4B). Volicitin treatments of 10, 30, 100, and 500 pmol resulted in sesquiterpene volatile emission of 1.3, 1.7, 4.8, and 5.8 μg 4 h^{-1}, respectively (Fig. 4B). Thus a positive relationship exists between the amount of volicitin applied

to a wound site, increases in JA levels, and resulting volatile emission.

Volicitin-ethylene interaction

Insect herbivory has been shown to induce ethylene production in multiple systems, yet numerous preliminary trials looking for volicitin-induced ethylene production failed to demonstrate differences. To increase the chance of detecting a change, plants were treated 4 times over the course of 20 h, and then examined for ethylene production. Mean ethylene production from untreated controls, mechanical damage + buffer, and mechanical damage + volicitin treatments did not significantly differ and averaged between 1.44 and 1.47 nl g^{-1} h^{-1} (Fig. 5). In contrast, BAW herbivory, a positive control for ethylene induction, resulted in a 4-fold increase in ethylene production above all other treatment groups (Fig. 5). Given that induced-ethylene production occurs during insect herbivory but not following treatment with volicitin, we examined the interaction of the two components. Low levels of atmospheric ethylene greatly increased both indole and sesquiterpene volatile emission from intact plants treated with volicitin. On a mean basis, volicitin resulted in greater sesquiterpene emission than comparable plants treated with buffer alone. However, this result was not statistically significant (Fig. 6A). Incubated overnight in the presence of ethylene at 0.25, 0.5 and 1 μl l^{-1}, volicitin induced sesquiterpene emission increased by 3.4-, 5.8-, and 6.4-fold, respectively (Fig. 6A). Ethylene also had a dramatic effect on indole emission. Additions of 0.25, 0.5 and 1 μl l^{-1} ethylene resulted in 74-, 93-, and 182-fold increases in volicitin induced indole levels (Fig. 6B). Interestingly, in both cases, ethylene did not significantly promote wound induced volatile emission (Fig. 6A,B)

JA-ethylene interaction

Atmospheric ethylene increases the sensitivity and magnitude of the intact plant response to exogenous JA. In the absence of additional ethylene, 50 nmol plant^{-1} JA
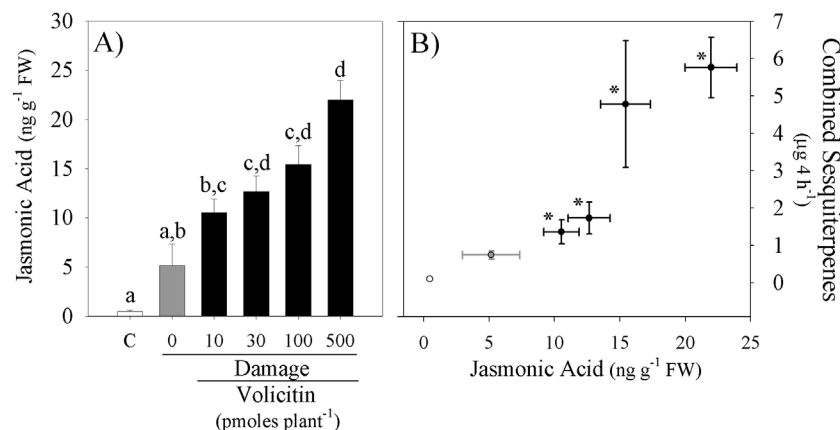

Fig. 4. Mean (± SEM, n = 4) JA concentration (A) of excised-leaves left as untreated controls (c) or damaged and treated with buffer containing either 0, 10, 30, 100 or 500 pmol of volicitin. Relationship between volicitin-induced JA levels and resulting combined sesquiterpene emission (B). Bars not sharing the same letters (a-d) represent significant differences in JA levels (P < 0.05, Tukey correction for multiple comparisons). Asterisks denote significant increases in volatiles above the undamaged control (P < 0.05, using Dunnett's test for defined multiple comparisons).
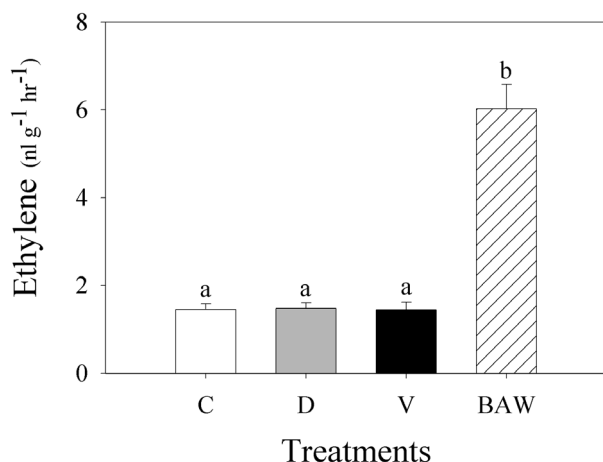
Fig. 5. Mean ($+$ SEM, n $=4$) ethylene production (nl g^{-1} h^{-1}) from intact plants left as untreated controls (C) or multiply damaged (4$\times$) over a 20-h period and treated with buffer (D) or buffer containing volicitin (1 nmol plant^{-1}) (V). A positive control for induced ethylene production consisted of plants infested with beet armyworm caterpillars (BAW). Bars not sharing the same letters (a-b) represent significant differences ($P < 0.05$, Tukey correction for multiple comparisons).

induces both sesquiterpenes and indole volatiles. However, 5 nmol plant^{-1} JA is inactive (Fig. 7A,B).

In the presence of 1 µl l^{-1} ethylene, the activity of 5 nmol plant^{-1} JA becomes equivalent to plants treated with 50 nmol plant^{-1} JA with no additional ethylene (Fig. 7A,B). The combined treatment of 50 nmol plant^{-1} JA and 1 µl l^{-1} ethylene produced 2.4- to 3.5-fold greater amounts of sesquiterpenes and indole than comparable plants without additional ethylene (Fig. 7A,B). At the concentrations used, added ethylene did not interact with mechanical damage alone to alter wound induced volatile emission.

Discussion

In this study we demonstrate that volicitin, a component of BAW OS, stimulates an increase in both JA and volatile emission when applied to mechanically damaged leaves of intact plants. While significant, the induction of volatile emission by volicitin on intact plants is modest. In comparison, the volatile responses of excised leaf and intact plant bioassays demonstrate that leaf excision increases the tissue sensitivity and magnitude of volatile induction following applications of both volicitin and JA. However, volicitin-induced JA levels do not differ between intact and excised assays; thus, JA levels alone do not readily explain the quantitative differences in volatile emission between bioassay designs. Relationships between volicitin-induced JA and resulting volatile emission were examined using the highly sensitive excised leaf assay. Over a constant level of mechanical damage, increasing applications of volicitin (10–500 pmol plant^{-1}) resulted in stepwise increases in both JA and sesquiterpene volatile emission. The positive relationship
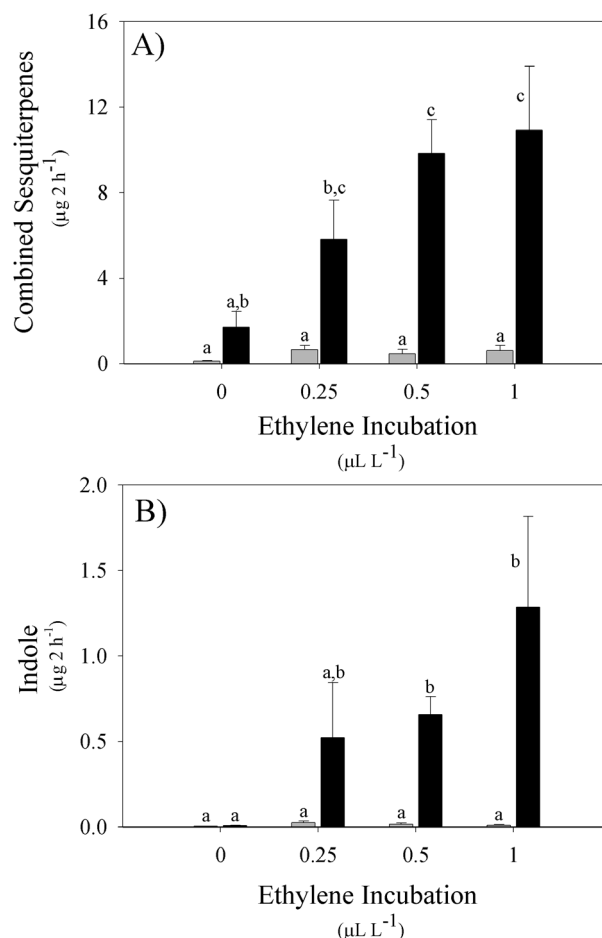

Fig. 6. Mean ($+$ SEM, n $=6$) volatile emission of combined sesquiterpenes (A) and indole (B) from intact plants following leaf damage treatments with either buffer alone (white bars) or buffer $+$ 1 nmol of volicitin (dark bars). All plants were incubated overnight in sealed containers containing either 0, 0.25, 0.5, or 1 µl l^{-1} of atmospheric ethylene. Within each figure bars not sharing the same letters (a-c) represent significant differences ($P < 0.05$, Tukey correction for multiple comparisons).

between volicitin-induced JA levels and resulting sesquiterpene volatiles supports the hypothesis that volicitin triggers volatile emission by acting through the JA pathway. Unlike insect herbivory, repeated wounding and the application of volicitin did not result in an increase of ethylene production. Thus we combined volicitin treatments with low levels of atmospheric ethylene to examine the interaction of components that are present during herbivory. The addition of ethylene to intact plant bioassays greatly increased the volatile emission of sesquiterpenes and indole following both volicitin and JA treatments. This suggests that the volatile inducing activity of insect derived elicitors, such as volicitin, may be amplified during insect attack due to the interaction with herbivore-induced ethylene.

In plants it has been well demonstrated that mechanical damage results in the accumulation of JA, which in turn regulates defense gene transcription (Creelman and
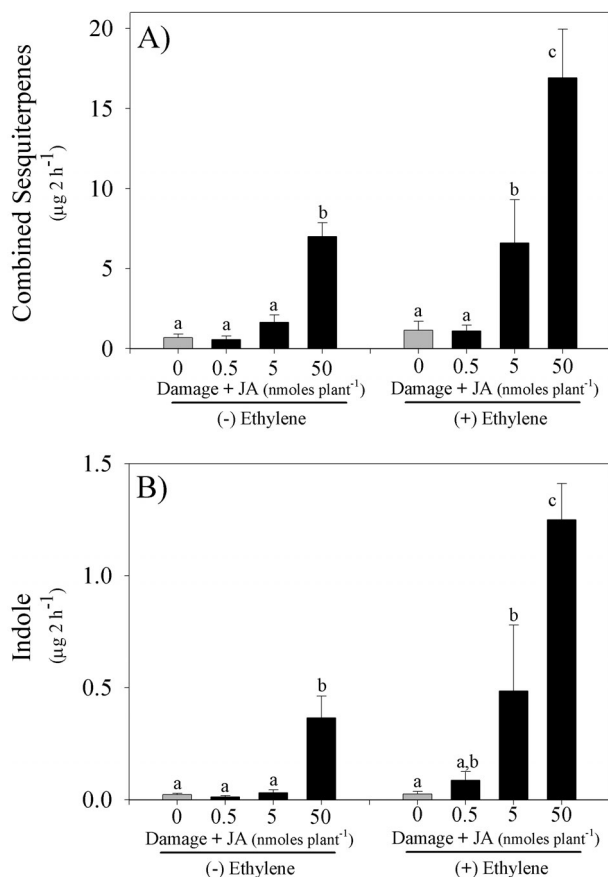
Fig. 7. Mean ($+$SEM, n $=$ 6) volatile emission of combined sesquiterpenes (A) and indole (B) from intact plants treated with leaf damage and buffer containing either 0, 0.5, 5.0, 50.0 nmol of jasmonic acid (JA). All plants were incubated overnight in sealed containers containing either 0 or 1 μl l^{-1} atmospheric ethylene, denoted as ($-$) and ($+$) ethylene, respectively. Within each figure bars not sharing the same letters (a-c) represent significant differences ($P < 0.05$, Tukey correction for multiple comparisons).

Mullet 1997). In addition to mechanical damage, plants also perceive and undergo specific responses to harmful biotic agents through the recognition of elicitors (Boller 1995). JA is believed to be an important mediator of plant responses to pathogen-derived elicitors present in bacteria (Brader et al. 2001) and fungi (Menke et al. 1999). Similarly, plants are known to respond differentially to wounding and insect herbivory (Baldwin 1988, Turlings et al. 1990, Reymond et al. 2000). These insect specific plant responses are believed to be due to the presence of insect derived elicitors and enzymes (Mattiacci et al. 1995, Alborn et al. 1997, Felton and Eichenseer 1999). Like many pathogen-derived elicitors, insect-derived elicitors have also been hypothesized to also act through the promotion of JA levels (Alborn et al. 1997, Paré and Tumlinson 1999). We now demonstrate for the first time that volicitin induces the accumulation of JA and that this increase corresponds with induced volatile emission. In an excised leaf bioassay, increasing amounts of volicitin applied to a constant level of mechanical

damage resulted in stepwise increases in volatile emission (Fig. 4). The amount of volicitin required for this response was in the range of 10–500 pmol plant^{-1} (4–211 ng plant^{-1}). In a careful examination of volicitin present in the OS of Noctuid caterpillars, Mori et al. (2001) found that levels averaged between 900 and 1400 ng larvae^{-1}. Unfortunately the actual quantity of elicitors contacting the plant leaf surface during a single bout of herbivory is unknown. Mindful of this gap in knowledge and the importance of staying within physiological relevance our experiments utilize levels of volicitin below those found in the OS of a single caterpillar. In a similar line of research, Koch et al. (1999) demonstrated that excised lima bean plantlets (*Phaseolus lunatus*) released volatiles consisting primarily of the homoterpene 4,8,12-trimethyltrideca-1,3,7,11-tetraene following the uptake of 2.0 μmol (812 μg) *N*-linolenoyl-L-Gln (18:3-Gln) per plantlet, a dehydroxy analogue of volicitin, from an aqueous solution. At comparable concentrations, free linolenic acid produced a similar volatile response in this system. Given that linolenic acid is precursor to JA, it was hypothesized that 18:3-Gln may act by contributing free linolenic acid to the JA pathway after cleavage of the amide bond (Koch et al. 1999). We believe that the amount of volicitin used in our work is below the level required for the substrate-supply model proposed by Koch et al. (1999). To test this further we used ^{13}C-labelled 18:3-Gln to induce volatiles and searched for ^{13}C incorporation into the induced JA pools. Compared to volicitin, identical patterns of both induced JA and volatile emission were generated, yet we found no evidence for ^{13}C incorporation into the induced JA pools (data not shown). This finding supports the recent results of Halitschke et al. (2001) that insect derived fatty acid-amino acid conjugates are elicitors of JA and not precursors.

Given the complex spatial, temporal, and oral nature of insect herbivory, it may be unrealistic that a single elicitor or treatment can fully replicate the stimulus of insect feeding. In addition to changes in JA levels, increases in ethylene production occur within 8 h of BAW caterpillar feeding during the photophase (Schmelz et al. 2003) and this appears to be a common result of insect herbivory (Kendall and Bjostad 1990, Kahl et al. 2000). In our trials, only BAW herbivory significantly increased ethylene production, whereas volicitin treated plants and controls were identical (Fig. 5). The known presence of induced-ethylene production during herbivory and the absence of ethylene following volicitin treatment prompted us to examine the possible interaction. The overnight addition of ethylene to both volicitin and JA treated plants synergized the volatile emission of intact plants. The release of volatile indole, following treatment with both volicitin and ethylene represents the clearest example of this interaction (Fig. 6B). Ethylene at 0.5 μl l^{-1} promoted over a 100-fold increase in the volicitin-induced indole emission when plants were treated in the evening and volatiles were collected the following morning. The significance of both JA and ethylene is also supported by recent experiments with an ethylene

perception inhibitor, 1-methylcyclopropene (1-MCP), and BAW herbivory in maize seedlings. Plants pre-treated with 1-MCP displayed 2- to 4-fold decreases in sesquiterpene and indole emission, respectively, when compared to similarly infested controls (Schmelz et al. 2003). We propose that the lack of ethylene production from intact plants treated with volicitin may explain the reduced activity of this elicitor compared to the greater plant responses witnessed following insect herbivory.

The synergistic interaction of jasmonates and ethylene in promoting the expression of plant pathogen defenses has been previously described. In tobacco, ethylene and methyl jasmonate are known to synergistically induce mRNA levels of specific pathogenesis-related (PR) proteins (Xu et al. 1994). Likewise in *Arabidopsis*, the simultaneous presence of both ethylene and jasmonate is required to synergize the accumulation of the plant defensin (PDF1.2) mRNA levels (Penninckx et al. 1998). Not surprisingly the combined effects of ethylene and JA on gene transcription can vary greatly and range from synergistic to antagonistic (Reymond and Farmer 1998, Pieterse and van Loon 1999). For example, when specialist caterpillars such as *M. sexta* feed on wild tobacco (*Nicotiana attenuata*), the insect-induced ethylene burst is believed to partially inhibit the wound induced nicotine accumulation that is regulated by JA (Kahl et al. 2000). Here, ethylene depresses the accumulation of putrescine *N*-methyltransferase mRNA, which corresponds to a key enzyme in wound and JA-induced nicotine biosynthesis (Winz and Baldwin 2001). In maize seedlings, we find a positive interaction of ethylene with both volicitin and JA on induced volatile emission (Figs 6,7). In tomato wound responses, ethylene is believed to regulate the level of JA that is accumulated following mechanical damage (O'Donnell et al. 1996). Interestingly, the ethylene perception inhibitor 1-MCP did not influence the induced accumulation of JA in maize seedlings attacked by BAW larvae (Schmelz et al. 2002). Future work will closely examine the potential influence of ethylene on volicitin-induced JA levels.

Our recent results and the work of others (Hopke et al. 1994, Halitschke et al. 2001) support a role for JA in regulating insect-induced volatile emission. Yet JA alone does not explain the large differences in volicitin-induced volatile emission witnessed when excised leaves and intact plants are compared. Excision may alter either the levels of, or sensitivity to, other unmeasured hormones. One well-documented difference between excised leaves and intact plants is ethylene production and or sensitivity (Morgan et al. 1990, Tsai et al. 1996). In bean and cotton leaves, rapid drying promoted by excision resulted in increased ethylene emission while the drying of intact plants did not (Morgan et al. 1990). Alternatively, ethylene sensitivity in excised tissues can be enhanced without actual increases in production (Tsai et al. 1996). In maize seedlings, other major stresses such as nutrient deficiency are known to dramatically enhance ethylene sensitivity without increasing production (He et al. 1992). This may also be of significance as excised leaf bioassays are commonly performed in vials of purified H_2O devoid of additional nutrients. Numerous preliminary experiments were performed on both intact plants and excised leaves designed to detect either wound or volicitin-induced increases in ethylene production. To date we have no evidence to suggest that either excision or volicitin treatments increase ethylene production in maize seedlings. However, given these negative results, we consider excision-induced changes in ethylene sensitivity as a possible mechanism to explain the differences in volicitin-induced volatile emission between bioassay designs. Clearly, the role of ethylene production or sensitivity in regulating volatile emissions in maize is worthy of a closer examination.

In maize seedling bioassays utilizing excised tissues, volicitin acts as a dramatic inducer of volatiles (Alborn et al. 1997, Frey et al. 2000, Schmelz et al. 2001). Volicitin is also active when applied to the wounded leaves of intact growing plants. However, the change in volatile emission is of a much smaller magnitude. Shortly after the discovery of volicitin it was envisioned that such insect-derived elicitors could be used as 'reagent grade' herbivores (Baldwin and Preston 1999), in essence, ecological research tools used to avoid the inherent variability associated insect feeding behaviour when investigating the role of plant responses in plant–insect interactions. Since this time, such optimism has been tempered by the modest activity of volicitin on intact plant systems (Schmelz et al. 2001). It now appears that other plant hormones modulated by insect damage, such as ethylene, interact with volicitin and induced JA pools to result in the high levels of volatile emission characteristic of insect herbivory. The consideration of plant hormonal changes stimulated by both insect-derived elicitors and insect attack is now beginning to elucidate why plant responses following mechanical damage are different from herbivory.

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